Investigating differing aquaria environments and their influence on natural behaviours and breeding patterns of captive-bred short-snouted seahorses, *Hippocampus hippocampus* (Linnaeus, 1758)

Stefanie Françesca Lamb

Msc Sustainable Aquaculture Systems

School of Biological Sciences, University of Plymouth, PL4 8AA

Abstract

The short-snouted seahorse (*Hippocampus hippocampus*) is one of two native seahorse species found in coastal British waters. Despite being fully protected by the Wildlife and Countryside Act (1981) and CITES (2003), they are still currently under threat as a result of anthropogenic sources including; by-catch, habitat degradation and the International Aquarium Trade (IAT). The past ten years has allowed for a greater amount of knowledge and experience to be gained regarding the biology and behaviours of captive-bred *Hippocampus spp*., nevertheless the amount of information currently available is still very limited. This pilot study therefore demonstrates that by improving and enhancing captive conditions in providing a more natural environment within the aquaria, allowed the captive-bred *H.hippocampus* to show natural behaviours. Diurnal activity and daily patterns, as well as beginning to demonstrate early signs of courtship displays were examples of this. This study has provided an insight for future work which could be carried out with regard to *H.hippocampus* and improving captive conditions, increasing conservation efforts and providing enhanced fish welfare.

Keywords: *Hippocampus hippocampus*, Aquaria, Behaviour, Competition, Courtship

Introduction

Globally, numerous species of seahorse are becoming increasingly vulnerable due to anthropogenic, physiochemical and biological factors (Foster & Vincent 2004, Planas *et al.* 2008, Koldewey & Martin-Smith 2010) which are continuously causing the marine environment to change. Seahorses occupy shallow, sheltered coastal habitats (Hilomen-Garcia *et al.* 2003, Murugan *et al.* 2009) which is putting them under greater threat as these areas of the oceans are being affected predominantly

by human activities. Incidental and direct by-catch, overfishing, TCM (Traditional Chinese Medicine), IAT (International Aquarium Trade) and habitat degradation (Murugan *et al.* 2009, Garrick-Maidment *et al.* 2010, Koldewey & Martin-Smith 2010) are the major causes attributed to these wild seahorse populations threatened status. Due to their low mobility, sparse distributions, low fecundity rates and small home ranges it has made these unique organisms particularly vulnerable (Garrick-Maidment 1997, Pinnegar *et al.* 2008, Otero-Ferrer *et al.* 2010, Woodall 2012), and has thus resulted in 33 out of 46 of these seahorse species to be included in the IUCN (International Union for Conservation of Nature and Natural Resources) red list (2006). *Hippocampus hippocampus* and *Hippocampus guttulatus,* two species of seahorse which are found native to British waters are both included in the IUCN red list. As of 6th April 2008 however, both of the native populations of British seahorse species are now considered to be fully protected by the Wildlife and Countryside Act 1981 and CITES 2003 (Convention on International Trade in Endangered Species of Wild fauna and Flora) which prohibits the killing, injuring or taking by any method of these wild organisms as well as international trade.

Short-snouted seahorses (*Hippocampus hippocampus*) are distributed along the southern coast of England, with substantial populations located around the Channel Islands and Ireland. Populations however have also been recorded previously along the coastlines of France, Belgium, Greece and Holland (Garrick-Maidment 1997, Garrick-Maidment & Jones 2004). Compared to *Hippocampus guttulatus*, *H.hippocampus* lack appendages and are smaller with an average length of around 12.5cm. They vary in colour from browny-orange to purple or black, providing them with effective camouflaging abilities (Garrick-Maidment 1997, Garrick-Maidment *et al.* 2010). Being smaller in size allows *H.hippocampus* to occupy a variety of habitats ranging from weedy algal areas such as eelgrass beds, silt and sediment environments to rocky macro-algae forests (Sabatini & Ballerstedt 2007). Although *H.hippocampus* are usually found in shallow waters, during the winter period they will migrate to deeper waters in order to escape the ferocity of the sea and waves in the shallows. They migrate back to the shallows in spring which offers greater protection and allows them to breed (Garrick-Maidment 1997, Sabatini & Ballerstedt 2007, Pinnegar *et al.* 2008).

Due to the unique structure of *Hippocampus spp.*, locomotion occurs via dorsal and pectoral fin undulation. This results in slow speeds and high manoeuvrability

which is advantageous for mating, predation, and predator avoidance within a complex, obstacle-strewn environment such as eelgrass beds and coral reefs (Garrick-Maidment 1997, Consi *et al.* 2001, Warfe & Barmuta 2004, Garrick-Maidment & Jones 2004). This type of slow motion movement is beneficial to seahorses when they must link up and mate as well as enabling them to effectively camouflage themselves from predators (Foster & Vincent 2004, Curtis & Vincent 2006, Koldewey & Martin-Smith 2010).

Identifying behaviours expressed by seahorses in both the wild and in captive environments is crucial in order to better help our understanding and ability of rearing *Hippocampus spp.* in aquariums. Although information with regards to seahorse biology and behaviours has increased in the past 10 years (Foster & Vincent 2004, Planas *et al.* 2008, Lucas & Southgate 2012), there is still a severe lack of knowledge concerning the majority of *Hippocampus spp*. Rearing seahorses in captivity however has begun to provide an insight into aspects of their everyday life and certain behavioural patterns such as; mate choice, reproductive periods and life cycles (Wilson & Vincent 1999, Storeo & Gonàlez 2009, Garrick-Maidment *et al.* 2010). In the wild, the duration of the breeding season for seahorses can begin from as early as April and continue through until October. However this varies with each species for example *H.hippocampus* the season is slightly shorter due to influencing factors which include; length of available daylight hours, and the temperature and turbulence of the water (Lourie *et al.* 1999, Woods 2000). During the breeding season, the majority of *Hippocampus spp.* (which includes *H.hippocampus*) will demonstrate a single breeding cycle with a monogamous relationship (Garrick-Maidment *et al.* 2010), though this may vary amongst species (Kvarnemo & Ahnesjo 1996, Woods 2000, Naud *et al.* 2008).

This investigation aims to observe the daily behaviours and patterns of captive-bred *H.hippocampus* in three differing environments to distinguish if there are alternative behaviours expressed dependent upon the surrounding environment of the aquaria. Throughout this investigation the potential for captive-bred *H.hippocampus* to successfully breed will be observed. Previous research has been conducted with regards to varying species of *Hippocampus* and indications are that by enhancing the aquaria to provide a more “natural” environment, has allowed for more natural behaviours (as seen in wild species) to be displayed, such as; daily

patterns, male aggression, courtship displays and successful breeding (Vincent 1990, Faleiro *et al*. 2008).

Materials and Methods

Profile of experimental *H.hippocampus*

Nine individual short-snouted seahorses were utilised for this investigation; six males and three females stocked in a 2:1 ratio. The *H.hippocampus* were bred in captivity (June 2010) at London Zoo (ZSL) and were then transferred to the National Marine Aquarium (NMA) in May 2011, ageing them at around 3 years old. Prior to the investigation, the seahorses were maintained in several aquaria under identical and sterile conditions containing no substrate and thick netted green rope structures attached to individual weights to act as holdfasts (Figure 1). Each aquaria had a volume of 136L (57x48x54cm) and was connected to the same re-circulating system. The seahorses were fed three times a day (10am, 12pm and 3pm) and subject to a 10% daily water change.

H.hippocampus identification

Individual seahorses were classified by their identity tags which were numbered and placed loosely around their necks with red elastic cotton. The tags were used to ease identification and to allow individual behaviour patterns to be monitored. Each of the three aquaria contained two male seahorses and one female, chosen purely at random due to the number of available seahorses kept at the National Marine Aquarium. The chosen seahorses were placed into the different aquaria at random, ensuring the 2:1 ratio was maintained. The initial set-up of the investigation commenced with; M17, F3, M13 in aquaria one (Figure 1), M20, F2 and M46 in aquaria two (Figure 2) and M08, M56, and F1 in aquaria three (Figure 3). M and F denote whether the seahorse is male or female. The seahorses were then rotated around the three aquaria in a clockwise direction every 22 days for a 66-day period: 1→2→3, 2→3→1 and 3→1→2.

Experimental aquaria and maintenance

The duration of this project was carried-out at the NMA in Plymouth, where the captive-bred *H.hippocampus* were maintained in a re-circulating system comprising

of three aquaria, each with a volume of 136L (57x48x54cm). Constant water parameters and aeration were maintained throughout the investigation supplied via the air line and inflow pipes seen in each of the three aquaria: salinity 20‰, pH 7.8-8.0, and temperature 16-17°c. The salinity was kept below the usual average level (34‰) for part of the investigation (28th May – June 22nd) due to the presence of a zoonotic disease outbreak occurring before the investigation commenced. By carrying out a hypo salinity treatment and maintaining the salinity at a constant 20‰ for a period of 6 weeks prior to (and during) the commencement of the investigation, it aimed to prevent the presence of this mycobacterium from returning to the system. The filtration method operating in this closed recirculating system contained;

- A biological filtration system with maturing bio-balls, which allows for the transformation of toxic waste materials (primarily ammonia) into relatively non-toxic nutrients through the activity of live micro-organisms. This method however does not remove the waste completely; water changes were therefore carried out periodically (Monday and Friday).
- Foam fractionation or Protein skimming. An effective method of chemical filtration that helps maintain aquariums as it allows the dissolved organic compounds (DOC) which are in the salt water to become adsorbed to the interface between the air and water. The air is injected into the vertical fractionator column, forming fine bubbles which then rise up creating a surface foam which is collected, removed and emptied periodically allowing for greater water clarity and cleanliness within the aquaria (Moe 1992, Lucus & Southgate 2012). This is advantageous as these DOC's can't be removed by normal mechanical filtration systems.
- Ultraviolet lighting was used to decrease the abundance of free floating bacteria and to control parasitic infections by killing the organisms during the free swimming stage of their life cycle.

During the investigation, the levels of nitrite (0.1 mg^{-1}), nitrate (10.0 mg^{-1}) and ammonia (0.1 mg^{-1}) in the water were all kept below the level at which they can become harmful to marine organisms.

Each of the three aquaria were placed side by side, and in order to eliminate any influence on behaviour from the neighbouring aquaria, the panels on both the left and right sides and the back were all blacked out. The environment inside the first

Figure 1: Aquaria one design and set-up involving two thick netted green rope structures each attached to individual weights with no substrate.

Figure 2: Aquaria two design and set-up involving a stony-gravel substrate and two artificial branching reed plants each attached to individual weights.

Figure 3: Aquaria three design and set-up involving a stony-gravel substrate with live macro algae (*Caulerpa prolifera*) utilised as the holdfast provided.

aquaria contained two pieces of thick netted green rope each attached to separate weights (Figure 1). The second aquaria contained a substrate consisting of stony-gravel and two artificial branching reed plants (Figure 2). The final aquaria again contained a substrate consisting of a stony-gravel and *Caulerpa prolifera* (Figure 3). *Caulerpa* was chosen as it has a similar structure (long-leaved and sends out runners) to that of eelgrass which wild species of *Hippocampus* utilise as a holdfast in certain areas of British and European coastlines (Garrick-Maidment 1997).

Feeding occurred routinely three times a day; 10am, 12pm and 3pm ensuring at least a period of 1hr30 between each feed. The seahorses were fed on frozen mysid shrimp (*Americamysis bahia*) in a salt-water solution with vitamins and minerals (VishVits; vitamins A, E and D3) substituted into their feeds twice a week (Monday and Thursday). To ensure hygienic conditions were constantly maintained within the aquaria, they were subject to a 10% daily water change, with aquaria one (containing netted rope structures and no substrate) being cleaned by daily siphoning, and aquaria two and three being siphoned every other day.

The lighting system used in the aquaria was AquaRay AquaBeam LED 12 watt in marine white and were timed to have 10 minute periods where lighting increased and decreased to stimulate dawn and dusk. These times were altered on a weekly basis in order to coincide with the sunrise and sunset rhythms of Plymouth which can be seen in table 1. The general maintenance and routines adopted for this investigation (regarding feeding times, cleaning, siphoning, lighting and water parameters) were identical to the conditions used prior to the investigation in order to prevent any notable changes from occurring within each of the aquaria and to ensure the data collection could be as reliable as possible.

Table 1: Weekly lighting time changes for sunrise and sunset to stimulate both dawn and dusk periods for Plymouth, Devon.

Date (2013)	Sunrise	Sunset
27th May	5:15	21:14
3rd June	5:09	21:21
10th June	5:06	21:27
17th June	5:05	21:31
24th June	5:06	21:32
1st July	5:10	21:31
8th July	5:16	21:28
15th July	5:23	21:23
22nd July	5:31	21:15
29th July	5:41	21:05

H.hippocampus behaviour

Behavioural patterns were monitored via an ethogram (Table 2) which was created for the purpose of this investigation. The seahorses in each of the aquaria were monitored for 15 minute periods before and after each feed, (1hr30 per aquaria per day) for the duration of 66 days. The aquaria which was monitored first for each of the 15 minute periods was rotated on a daily basis in order to eliminate bias. The seahorses in each of the aquaria were also rotated every 22 days to ensure that they would be exposed to each of the differing environments to investigate whether the aquaria set-ups were influencing their behavioural patterns. The same seahorses remained together throughout the duration of the trial.

Data collection

Data recordings of time spent swimming or at rest were taken for a period of 15 minutes on each of the separate aquaria six times per day, before and after each feed (9:00am, 10:15am, 11:15am, 12:15pm, 2:15pm, 3:15pm), six days per week for a total period of 66 days. These 15 minute time periods were recorded in seconds for ease of data input and analysis in Microsoft Excel and Minitab 16. This data was recorded every second over the 900 second time frame. Observations of data such as; holdfast attachments, male aggression, courting behaviours and body or head movements (as seen in table 2) were recorded as qualitative data.

Table 2: A behavioural ethogram representing the movements displayed by *H.hippocampus* and the type of holdfast attachments possible within each of the 15 minute periods of observations carried out for each of the individual aquaria.

Movements	**Behaviour description**
Rest (R)	Stationary, body is immobile
Head movement (HM)	Body is immobile, slight head movements
Body movement (BM)	Slight body movements with dorsal and pectoral movements
Swimming (S)	Actively swimming in water column with dorsal and pectoral fins moving
Feeding (F)	Looking for or consuming food
Competition between seahorses (C)	Wrestling each other with their tails and body
Holdfast attachment	
Holdfast provided (HP)	Attached to the provided holdfast in the tank
Inflow pipe (IP)	Attached to the inflow pipe
Overflow pipe (OP)	Attached to the overflow pipe
Airline (AL)	Attached to the airline
Attached to other seahorses (AS)	Attached to another seahorse in the tank when at rest
Courting Behaviour	
Colour change (CC)	Change in the colouration of the seahorses (lightening)
Inflating pouch (IF)	Inflation of the pouch on the male seahorses
Tail holding (TH)	Seahorse grasping the tail of another seahorse when swimming
Vertical swimming as a pair (VS)	Pair of seahorses swimming in close proximity to each other vertically in the water column
Egg transfer (ET)	Transfer of eggs from female to male seahorse

Statistical analyses

Data which had been recorded for the quantity of time spent either swimming or at rest was transformed from the 15 minute periods into seconds. Body and head movements, feeding, courting behaviours and aggression were all recorded as a count over each of the 15 minute periods. Statistical analyses were then performed using a multi-level analysis of variance (ANOVA) on Minitab 16 which was used to evaluate; movements (swimming/rest), attachments and body and head movements between the differing aquaria over a 66-day period (Zar 1996). A significance level of $p<0.05$ was used for the multi-level ANOVA, as well as for the Kolmogorov-Smirnov test for homogeneity of variance. Following on from the multi-level ANOVA, Tukey's tests were carried out where significant results were found to test where the difference in the data was located.

Results

During the investigation one male seahorse (M46) died. This occurred on day 43, one day prior to when the seahorses were due to switch aquaria. Skin and gill scrape samples were taken from the deceased male and the cause of death is still under

histopathological investigation, however amoebic gill disease (AGD) is the suspected cause as this has caused issues previously in the NMA.

Before carrying out any statistical analyses, standard deviations for time spent swimming or at rest, and the number of body and head movements were calculated between the different seahorses in the three separate aquaria to ensure whether the data which had been collected could be included together (due to the fact that no replicates were carried out as a result of the availability of separate aquaria and seahorses present at the NMA). As there was some deviation away from the mean value for the individual seahorses in the separate aquaria, separate analyses were carried out due to the data points deviating slightly from the mean values (Dytham 2011). Once this had been done, a Kolmogorov-Smirnov test was carried out on the collected data from the investigation to see if the data was normally distributed. This test was used rather than the Shapiro-Wilk due to the large sample size of data. The results obtained from this showed that the data was not significantly different from the normal ($p>0.05$) so further statistical analysis could be carried out without the need to transform the data.

Influence of differing aquaria environments and observation times

Comparisons between individual seahorses, which aquaria they were in and the time of day the recordings were taken were compared on a multilevel ANOVA. Results obtained from this proved that variation in the quantity of time spent swimming in each of the three aquaria differed. M08 ($F_{2,5} = 6.62$ $p<0.05$), M56 ($F_{2,5} = 11.02$ $p<0.05$) M20 ($F_{2,5} = 3.85$ $p<0.05$), M46 ($F_{2,5} = 12.61$ $p<0.05$), F1 ($F_{2,5} = 5.07$ $p<0.05$) and M13 ($F_{2,5} = 62.29$ $p<0.05$) all showed statistically significant differences between recordings taken from each of the separate aquaria (Table 3, Figure 4). Variations between the times of the day at which the seahorses spent swimming was also proven to be statistically significant showing that the quantity of time the seahorses spent swimming varied through-out each day (Table 3); M56 ($F_{2,5} = 11.02$ $p<0.05$), M08 ($F_{2,5} = 6.62$ $p<0.05$), M13 ($F_{2,5} = 62.29$ $p<0.05$) and M17 ($F_{2,5} = 4.74$ $p<0.05$). Following on from this, another multilevel ANOVA was performed (Table 4) comparing the number of body and head movements which each seahorse made throughout the day, and in varying aquaria.

Table 3: Multi-level ANOVA showing the statistical significance and difference between individual *H.hippocampus* and their behavioural response (with regards to time spent swimming) to differing aquaria environments and at different times of the day.

Seahorse	*F* value	Aquaria	Time of Day
M17	$F_{2,5}$ = 4.74	p = 0.293	p = 0.018
Female 3	$F_{2,5}$ = 2.75	p = 0.112	p = 0.196
M13	$F_{2,5}$ = 62.29	p = 0.03	p = 0.001
M08	$F_{2,5}$ = 6.62	p = 0.015	p = 0.008
M56	$F_{2,5}$ = 11.02	p = 0.03	p = 0.002
Female 1	$F_{2,5}$ = 5.07	p = 0.03	p = 0.059
M20	$F_{2,5}$ = 3.85	p = 0.05	p = 0.945
Female 2	$F_{2,5}$ = 1.26	p = 0.325	p = 0.732
M46	$F_{1,5}$ = 12.61	p = 0.016	p = 0.604

Significant results were again displayed with M46 ($F_{2,5}$ = 38.9 p<0.05), M20 ($F_{2,5}$ = 9.81 p<0.05) M17 ($F_{1,5}$ = 3.26 p<0.05) and F1 ($F_{1,5}$ = 3.18 p<0.05) all showing that the number of body or head movements made varied in relation to which aquaria the recordings were taken in. Differences between the time of day recordings were taken and the number of body or head movements observed for each individual seahorse when attached to the holdfasts also proved to be statistically significantly different (Table 4, Figure 5); M20 ($F_{2,5}$ = 9.81 p<0.05), M46 ($F_{2,5}$ = 38.9 p<0.05) and M56 ($F_{2,5}$ = 4.80 p<0.05).

Table 4: Multi-level ANOVA showing the statistical significance and difference between individual *H.hippocampus* and their behavioural response (with regards to body and head movements when attached to their preferred holdfast) to differing aquaria environments and at different times of the day.

Seahorse	*F* value	Aquaria	Time of Day
M17	$F_{2,5}$ = 3.26	p = 0.025	p = 0.081
Female 3	$F_{2,5}$ = 2.88	p = 0.072	p = 0.28
M13	$F_{2,5}$ = 4.05	p = 0.29	p = 0.192
M08	$F_{2,5}$ = 1.62	p = 0.240	p = 0.581
M56	$F_{2,5}$ = 4.80	p = 0.063	p = 0.035
Female 1	$F_{2,5}$ = 3.18	p = 0.05	p = 0.77
M20	$F_{2,5}$ = 9.81	p = 0.019	p = 0.041
Female 2	$F_{2,5}$ = 0.8	p = 0.56	p = 0.68
M46	$F_{1,5}$ = 38.9	p = 0.012	p = 0.002

The final multilevel ANOVA which was carried out was to show the differences seen between holdfast preference within each aquaria, and whether this varied at certain times of the day. Prior to this being undertaken, the standard deviation for the raw data was analysed to see whether this data could be included together. The deviation from the mean had little variation between the male seahorses so these could be included together. The females also had minimal variation between each

other, therefore allowing the data to be collated together. However, the females varied significantly from the males so the males and females were analysed separately. The results obtained for the females showed that there was no significant difference ($F_{1,5}$ = 6.07 p>0.05) in the preference of holdfasts at certain times of the day, however there were significant differences ($F_{1,5}$ = 6.07 p<0.05) seen between the different aquaria (Table 5). The males also appeared to show the same differences as the females with a significant difference seen between the differing aquaria ($F_{1,5}$ = 9.93 p<0.05) but not between different times of the day ($F_{1,5}$ = 9.93 p>0.05). These results have shown that both the male and female *H.hippocampus* utilised in this pilot investigation preferred certain holdfast over others when subject to the different aquaria environments.

Table 5: Multi-level ANOVA showing the statistical significance and difference between male and female *H.hippocampus* and their behavioural response (with regards to holdfast preference) to differing aquaria environments and at different times of the day.

Seahorse	F value	Aquaria	Time of Day
Male	$F_{2,5}$ = 9.93	p =0.034	p =0.759
Female	$F_{2,5}$ = 6.07	p = 0.048	p =0.96

Variation between aquaria

As the p values were significant for the differences seen between the different aquaria regarding holdfast attachments for both male and female *H.hippocampus*, Tukey's tests were carried out on both of these multilevel ANOVA's to see which aquaria differed from the other. This is due to the fact that ANOVA's cannot distinguish differences between groups in the data. The results of this revealed that both aquaria two and three were significantly different from aquaria one but there was no difference between aquaria two and three. These results were seen for both the males and females with regard to different holdfast preferences between each aquaria. Tukey's tests were then carried out to see if there were differences between the significant results obtained from the multi-level ANOVA's with regard to time spent swimming and body and head movements. M13, M08, F1 and M20 all showed that there was a significant difference seen between aquaria one and aquaria two and aquaria one and aquaria three for time spent swimming in each, which figure 4 and figure 6 demonstrate. M20 and F1 showed that body and head movements were significantly different between aquaria one and aquaria two and aquaria one and

aquaria three (Figure 5) as well as M46 showing differences between aquaria two and aquaria three (Figure 5).

Variation between times of observations

The mean percentage of time spent swimming has shown differences between each of the times at which observations were made (9:00am, 10:15am, 11:15am, 12:15pm, 2:15pm, 3:15pm) regarding M17, M13 and M08 as there were statistical differences demonstrated by Tukey's test. Decreases in the amount of time spent swimming can be seen in figure 4 regarding the morning observations (9:00am) through to the afternoon (3:15pm). Tukey's test showed that M56 varied the time spent swimming throughout the day in each of the aquaria (Figure 4) with significant differences being seen between each of the three separate aquaria and at different times of the day. With regard to variation between times of the day and the mean number of body and head movements made, significant results from Tukey's test were obtained from M56, M46 and M20 (Figure 5) showing that a difference between the time of the day when recordings were taken and the mean number of body and head movements made varied significantly.

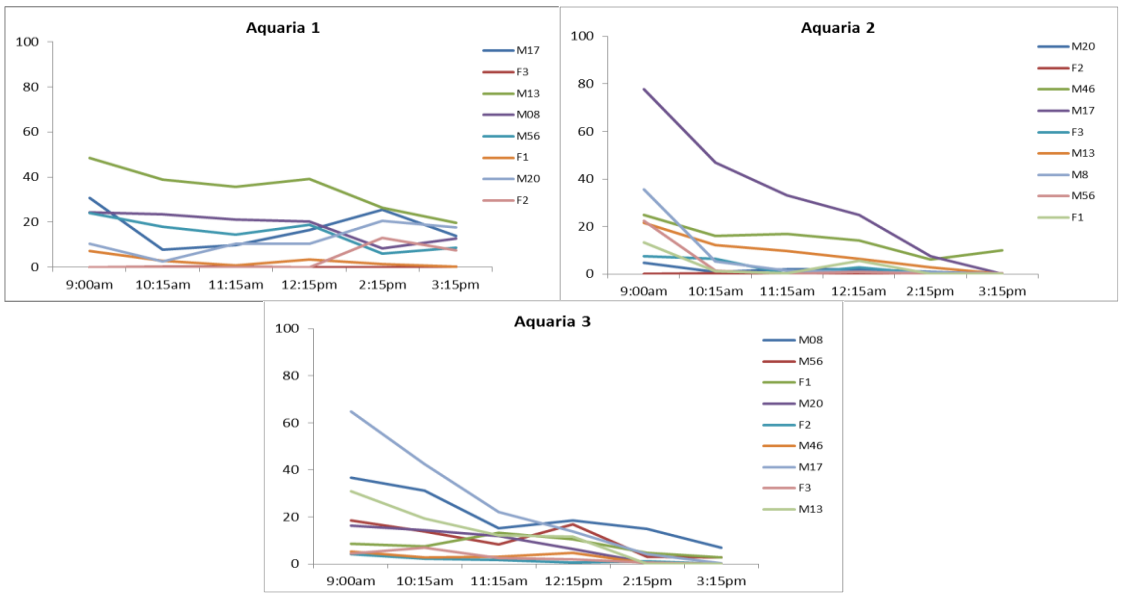

Figure 4: Mean percentage of time spent swimming for individual *H.hippocampus* in each separate aquaria (Aquaria one= no substrate and two pieces of thick netted green rope attached individually to separate weights, Aquaria two = stony-gravel substrate and two artificial branching reed plants attached to individual weights, Aquaria three= stony-gravel substrate and live macroalgae *Caulerpa prolifera*) over a 66 day period, six days per week, and at six specific times of the day; 9:00am, 10:15am, 11:15am, 12:15pm, 2:15pm & 3:15pm.

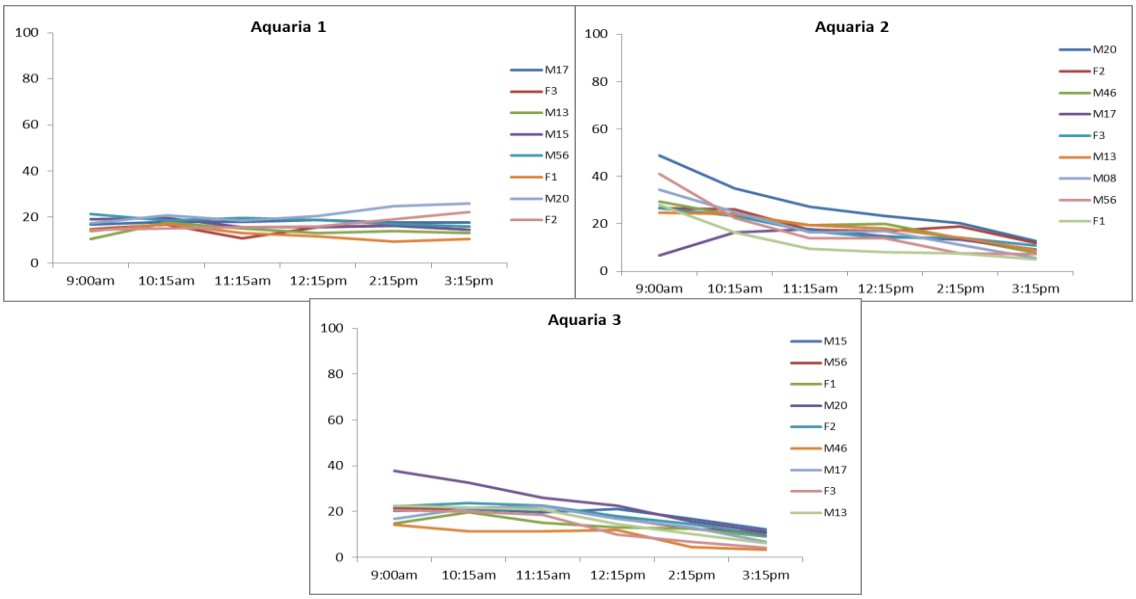

Figure 5: Mean number of body and head movements for individual *H.hippocampus* in each separate aquaria (Aquaria one= no substrate and two pieces of thick netted green rope attached individually to separate weights, Aquaria two = stony-gravel substrate and two artificial branching reed plants attached to individual weights, Aquaria three= stony-gravel substrate and live macroalgae *Caulerpa prolifera*) over a 66 day period, six days per week, and at six specific times of the day; 9:00am, 10:15am, 11:15am, 12:15pm, 2:15pm & 3:15pm.

Aggression and courting behaviour

Results which were obtained for male aggression and courting behaviour were recorded as qualitative data (Appendix 1, Table 2) as it enabled a more in-depth description to be recorded as opposed to quantitative data. Throughout the investigation, clear signs of male aggression were demonstrated by M20 and M46 in both aquaria two and three as well as M17 and M13 which also demonstrated aggression in both of these aquaria. M08 and M56 appeared to show more aggression in aquaria three than aquaria two. Signs of male aggression were recorded as either; wrestling with their tails (W), body barging each other (BB) or if they were “snapping” their snouts at the other male present in the aquaria (SS). No signs of male aggression were demonstrated in aquaria one with regards to any of the males. This qualitative data was recorded in Microsoft Excel throughout the 66-day period (Appendix 1).

Signs of courting behaviours were displayed in aquaria two and three, with M20 and M17 displaying the most encouraging signs. Each of these males showed signs on multiple occasions (in particular M20) by inflating their brood pouches with water (IF) and swimming slowly in front of the female present in the aquaria (F2 and F3) as well as M20 and F2 swimming together on several occasions. Both M20 and F2 were

also seen to be resting together on the same holdfast with their tails linked (AS). M20 inflated his pouch when first transferred from aquaria three to aquaria one, however after just three days in this aquaria the courting behaviour ceased. M17 did not display courting signs in aquaria one either.

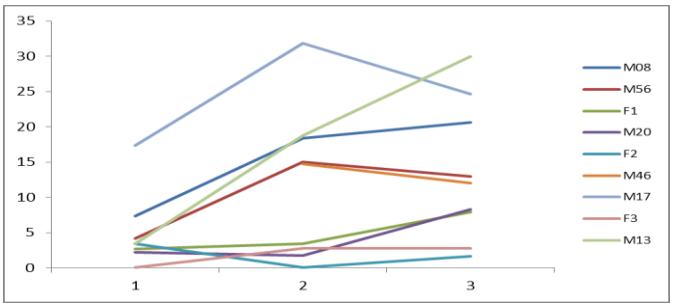

Figure 6: Average percentage of time spent swimming calculated over the whole 66 day period of observations for each individual seahorse in each of the separate aquaria (1=no substrate and two pieces of thick netted green rope attached individually to separate weights, 2= stony-gravel substrate and two artificial branching reed plant attached to individual weights, 3= stony-gravel substrate and live macroalgae *Caulerpa prolifera).*

Discussion

Daily patterns of *H.hippocampus*

During this investigation certain patterns in activity can be noted throughout the 66-day period of observations. The majority of the seahorses monitored for this investigation displayed much more active behaviour in the mornings as opposed to the afternoon where they became less active and spent the majority of their time at rest, attached to holdfasts (Figure 4, Table 3). This type of daily activity is notably common for wild species of *H.hippocampus* as they are displaying signs of diurnal activity (Garrick-Maidment 1997, Foster & Vincent 2004, Falerio *et al.* 2008).This form of diurnal activity is thought to be due to certain influencing factors such as; available light, which has been successfully adjusted and adapted for the *H.hippocampus* in the NMA by altering the lighting to display periods of dawn and dusk, prey availability and stocking densities (Moe 1992, Wong & Benzie 2003). All of these factors are particularly important to consider when breeding and rearing seahorses in captivity as in the wild, female *H.hippocampus* occupy a territory of about 1.4 square metres and males 0.5 square metres (Garrick-Maidment 1997). This therefore shows that by sparsely stocking the aquaria (as seen in this pilot

study) it will be advantageous for the seahorses as they will be less stressed (Schrek1990), therefore more inclined to display a variety of natural behaviours such as daily patterns and courting displays as they are able to advance/move away from each other in the aquaria (Foster & Vincent 2004, Koldewey & Martin-Smith 2010).

Hippocampus spp. are very sensitive to light, therefore by utilising the lower light levels available in the early mornings to forage, feed and engage in courtship displays has proven to be highly beneficial to them (Garrick-Maidment & Newman 2011). This is particularly true to *H.hippocampus* as they occupy habitats which have relatively low levels of light for example eelgrass beds, and sand or silt environments. This ability to see in low light levels influences their daily behaviours in the wild as they will feed largely at dawn when the presence of zooplankton such as copepods are abundant due to diel vertical migration (Loose & Dawdowicz 1994). Early morning feeding behaviours appeared to be more common in this pilot investigation whereby the *H.hippocampus* appeared to swim more actively to locate their food when fed in the morning compared to the afternoon feeds. Due to the presence of a controlled lighting system in the NMA, patterns demonstrated by the captive-bred *H.hippocampus* regarding feeding and foraging and being more active in the mornings shows how it is vital to mimic these dawn and dusk periods in captive environments in order to encourage more natural behaviours which are seen in wild *Hippocampus spp.* (Garrick-Maidment 1997, Faleiro *et al.* 2008, Murugan *et al.* 2009).

Wild *Hippocampus spp*. demonstrate more active behaviours in the mornings as a result of increased foraging and feeding, providing them with the energy requirements they need (Foster & Vincent 2004). Results obtained from this pilot investigation have agreed with previous reports by Garrick-Maidment and Newman (2011) regarding daily activities and movement patterns. The results obtained from this investigation have shown that captive-bred *H.hippocampus* decreased their time spent swimming as well as the number of head and body movements when attached to the provided holdfasts in the aquaria throughout the duration of the day (Table 3, 4 and Figure 4,5). Male *H.hippocampus* appeared to be more active throughout this investigation than the females (Figure 4 and 5), with M08, M56, M17 and M13 ($F_{2,5}$ = 6.62 p=0.008, $F_{2,5}$ = 11.02 p=0.002, $F_{2,5}$ = 4.74 p=0.018, $F_{2,5}$ = 62.29 p=0.001) showing significant differences between time spent swimming (Table 3, Figure 4) and resting compared to the females who didn't vary their activity patterns throughout the

day. This reduction in female activity could be attributed to the fact that in the wild the female *H.hippocampus* occupy a much larger territory than that of the aquaria which they were kept in. This lack of space could therefore be influencing the types of behavioural patterns which were observed by the female *H.hippocampus* in this investigation. Storero and González (2009) found that captive-bred seahorses appeared to spend at least 50% or more of their time in a static position in the morning followed by an increase in this percentage in the afternoons to allow for better prey capture. This type of behaviour noted by Storero and González appears to be similar to that of the behaviours of the female seahorses in this investigation as they appeared to spend a large proportion of their time at rest (Figure 4).

Courtship behaviours

Breeding and courting behaviours displayed by *Hippocampus spp.* have been seen to show four different phases of the courtship cycle before the transfer of the hydrated eggs occurs from the female to the male (Masonjones & Lewis 1996, Masonjones & Lewis 2000, Woods 2010). These four phases will usually transpire 2-3 days before the egg transfer occurs and involves the male seahorse moving rapidly from side to side when attached to a holdfast, followed by the female raising her head. The male then follows the female's actions and begins to raise his head up. The following day the seahorse pair will meet at dawn and repeatedly rise vertically in the water column, shortly to be followed by the transfer of the eggs (Masonjones & Lweis 1996, 2000). Courting signs however such as the inflation of the male brood pouch, swimming as a pair in the water column and colour changes have been seen to occur before this final four phase cycle takes place which is highly significant behaviour as the colour change signifies social interaction between the male and female, and pouch inflation indicates the male seahorses readiness to mate (Vincent 1990, Vincent 1995, Foster & Vincent 2004, Murugan *et al*. 2009).

Although breeding was unsuccessful during this 66-day period of investigation, early signs of courting behaviours (Masonjones & Lewis 1996) were seen on several occasions, particularly concerning M20 and M17. During the first two periods of monitoring (before morning feed, 9:00am and after morning feed, 10:15am) M20 displayed signs of courting towards F2 on several occasions when observed in aquaria two. When moved to aquaria three however the presence of early courting

signs were observed more frequently as well as M20's colouration lightening when swimming in front of F2. These early courting signs involved M20 filling his brood pouch with water in front of F2, showing off and presenting to her the size of his brood pouch (Figure 7). This courting behaviour was also noted when observing M17 in aquaria three. Although M17 did not inflate his brood pouch very often, and was only observed demonstrating this in aquaria three, this could still be demonstrating positive signs for future captive breeding programs of *H.hippocampus* with regard to the set-up of aquarium tanks. When first moved from aquaria three to aquaria one, M20 inflated his pouch only several times in the first few days but then these courting displays ceased for the final observation period (from day 48 to 66). This may have been a result of the more sterile "unnatural" environment set-up influencing M20's behaviour patterns. Conversely, M20's behaviour may have also been influenced by the death of M46, altering the pre-existing biased male sex ratio of 2:1 and changing it to 1:1 (Vincent 1994, Kvarnemo & Ahnesjo 1996, Naud *et al*. 2008).

Early courting signs and displays were observed throughout this pilot investigation; however these observations were only seen in either aquaria two or more often in aquaria three. This is therefore showing that the more naturally created habitats in the aquaria are providing a more appropriate environment for the *H.hippocampus* to potentially breed in the future and demonstrate behaviours and patterns similar to that of wild species.

Figure 7: Images depicting M20 inflating his brood pouch in aquaria three and M20 again inflating his brood pouch in the presence of F2 in aquaria one.

Male aggression

Male aggression can be viewed as interactions between several males in order to maintain or gain a dominant position over the opposing male seahorse by demonstrating competitive behaviour (Spence & Smith 2005, Faleiro *et al*. 2008).

Examples of this type of competitive behaviour have been seen in captivity with a variety of *Hippocampus spp*. regarding tail wrestling, body barging and snout snapping (Vincent 1990, Woods 2003, Foster & Vincent 2004). During this preliminary investigation, aggression between the male *H.hippocampus* was observed. These signs of aggression and competitive behaviours towards other males were observed only in aquaria two and three. M20 appeared to show the most competitive behaviours towards the other seahorse present in the aquaria (M46) as he demonstrated body barging and tail wrestling on many occasions before he began swimming around the female present in the aquaria. When M20 had inflated his brood pouch, he would often snap his snout aggressively towards the direction of M46 which then resulted in M46 swimming away from M20 and towards the other side of the aquaria. This type of male dominance behaviour demonstrates here that when unpaired male seahorses are influenced by a biased sex ratio (as seen in this pilot study), the more dominant male would appear to gain the advantage in pairing off with the female seahorse (Vincent 1994, Naud *et al*. 2008). Although this type of aggressive behaviour has been demonstrated here, and in other studies with regard to captive reared *Hippocampus spp*. it has been shown that this type of competitive behaviour may not exist in the wild and only in captivity due to the space restraints of the aquaria. This competitive behaviour was seen also with M17 and M13 during this investigation with M17 snapping his snout at M13 as well as tail wrestling occurring between the two. M17 appeared to be the more dominant and competitive seahorse compared to that of M13, as he showed courting signs whereas M13 did not. M08 and M56 both appeared to show aggression towards each other, however this was present largely in aquaria three compared to that of the other male seahorses who demonstrated this aggressive behaviour in both aquaria two and three.

During this investigation, male aggression was only viewed in aquaria two and three with no clear signs of aggression being observed in aquaria one. The group of seahorses containing M08, M56 and F1 showed no obvious signs of a dominant male unlike the other groups (M17, M13, F3) (M20, M46, F2), which could be linked to the fact that no courting behaviour was observed between these seahorses. Studies carried out by Faleiro *et al*. (2008) have shown that when male aggression and competition were displayed in captive environments it often interrupted courtship displays and the potential for seahorses to breed. This highlights the importance of

isolating *Hipocampus spp*. when they are beginning to show early courting signs in order to optimise the success of breeding seahorses in captivity in the future.

Holdfast preference

Holdfasts are crucial for *Hippocampus spp*. as they utilise these structures for resting, breeding, feeding and predator avoidance (Faleiro *et al*. 2008, Olivotto *et al.* 2008). Wild adult species of *H.hippocampus* will usually occupy vertical holdfast structures positioning themselves near the bottom so that they have a greater ability to camouflage themselves from predators as well as being able to locate their prey more easily (Foster & Vincent 2004). These observations have also been noted in previous studies with regards to differing species of seahorse (Faleiro *et al.* 2008, Woods 2010).

Variations throughout this investigation have been observed with regard to holdfast preferences and the environment in each of the aquaria (Table 5). A multi-level ANOVA and Tukey's test revealed significant differences between aquaria one and aquaria two and aquaria one and aquaria three for both male and female *H.hippocampus* ($F_{2,5}$ = 9.93 p= 0.034, $F_{2,5}$ = 6.07 p= 0.048) regarding holdfast preferences in the aquaria present. When *H.hippocampus* were present in aquaria one, they appeared to prefer to attach themselves to the holdfast provided (netted green rope, Figure 1) and position themselves on the same holdfast but apart from each other. They were also noted to be much higher up the holdfast structures than that of aquaria two and three where they positioned themselves at the bottom of the holdfast. When the seahorses were present in each of the other two aquaria (two and three) they would attach themselves to a variety of holdfast structures (airline, inflow pipe, overflow pipe, substrate) rather than just provided holdfast (Figure 2, Figure 3).

Wild *Hippocampus spp.* will attach onto a variety of structures and substrates when in the ocean rather than just the main holdfast present. For example, *H.hippocampus* which have been located in Studland Bay Dorset, Poole Harbour and Torbay Devon have been found attached to seagrass, macroalgae, small boulders, silt and sandy substrates as well as being found attached to anchor chains or discarded fishing nets (Garrick-Maidment *et al.* 2010, Garrick-Maidment 2011, Garrick-Maidment & Newman 2011). Variation in holdfast preference in aquaria two and three could therefore be demonstrating a more natural behaviour pattern similar to that of wild *Hippocampus spp.* as studies have proven that they appear to alter

what type of holdfast they attach themselves to, as well as certain species of seahorse preferring to utilise certain holdfast types (Perante *et al*. 2002, Woods 2003). Evidence which has been gained from previous work combined with the results from this pilot study could therefore provide useful information for future aquaria set-ups when concerning *Hippocampus spp*. and providing a variety of differing holdfast structures. However due to the fact that no replicates could be used during this pilot study as a result of *H.hippocampus* and aquaria availability at the NMA, highlights the need for future research to be carried out into this area of study.

Influence of captive environments

During this pilot investigation, promising signs of early courting behaviours were observed regarding male *H.hippocampus* when subject to aquaria two and three. However certain factors may have influenced the fact that breeding was not successfully observed. During the investigation, sunrise occurred at around 5:30am which is the usual period of the day when *H.hippocampus* will engage in courtship displays. Due to the early sunrise times, this meant that access to the NMA was unavailable, so observations could not be made then. Other influencing factors include the hypo salinity treatment which could have impacted upon the behaviours of the seahorses as well as compromising their ability to breed due to the possible effects of a stress induced response (Woods 2003, Koldewey & Martin-Smith 2010).

Exposure to noise has been shown to become deleterious to aquarium fish with regard to increased levels of stress resulting in decreased feeding rates and reproduction (Anderson 2013). The location of the aquaria used in this investigation was based in the NMA, with husbandry staff and engineers moving in and out of the room throughout the day. These movements combined with noise produced could therefore have influenced the behaviours and breeding success of the *H.hippocampus* being studied. Therefore with regard to improving breeding success of *Hippocampus spp*., future trials may try to eliminate as much noise as possible by utilising acrylic or concrete walls and providing substrate in the aquaria as shown by Bart *et al*. (2001) and Anderson (2013). The final factor which may have influenced these results were the diets (frozen *Americamysis bahia*) which were provided for the *H.hippocampus*. Although frozen diets will usually be enriched with highly unsaturated fatty acids (HUFA's), vitamins and minerals (Foster & Vincent 2004, Yin

et al. 2012), the freeze-thaw process will usually damage crucial components needed for growth and has been seen to cause a reduction in brood size and a decrease in breeding activity (Wilson & Vincent 1999, Koldewey & Martin-Smith 2010). It is therefore vital that live food should be supplemented into *Hippocampus spp*. feeds as they will contain higher levels of HUFA's which support improved membrane formation, osmoregulation and enhanced immune system function which has proven to increase seahorse growth rates and improve their chance of survival (Woods 2003, Olivotto et al. 2008, Yin *et al.* 2012), which will better provide for captive reared *Hippocampus spp.* in the future.

Conclusions

Results obtained from this preliminary study have concluded that captive-bred *H.hippocampus* appear to demonstrate daily behavioural patterns similar to that of wild species including; increased movements and time spent actively swimming as well as greater feeding and foraging during morning periods (9:00am and 10:15am). These observations highlight the importance of influencing light levels with regard to stimulating dawn and dusk periods within captive environments. The most significant outcomes from this investigation appear to have been seen in aquaria two and three, revealing that the more naturally created environments within the aquaria are positively influencing the *H.hippocampus* behaviours, allowing them to respond to the varying environmental parameters. Both aquaria two and three indicated signs of male aggression and competitive behaviours as well as M20 and M17 demonstrating courting behaviours towards F2 and F3 which involved brood pouch inflation, colour changes and circling slowly around the female present in the aquaria.

Despite the failed attempts to breed *H.hippocampus* successfully in this investigation, early signs demonstrated by M20 and M17 show that future breeding efforts may prove to be successful. The use of multiple holdfast structures was also demonstrated within aquaria two and three, showing the importance of variation and choice within captive environments in order to enhance aquaria and husbandry techniques. This study therefore highlights the importance of pilot investigations into the behaviours of *Hippocampus spp*. as it has allowed observations to be made ensuring that future captive environments will provide improved conditions for captive reared seahorses.

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Appendix 1:

Qualitative raw data entry

Observations made for aquaria three during the final 22 day period of observations showing the quantity of time spent at rest (15 minute periods recorded as seconds), quantity of time spent swimming (15 minute periods recorded as seconds), number of times *H.hippocampus* fed, signs of male aggression, number of body and head movements, holdfast attachment type and signs of courting behaviours.

Monday 22nd July 2013								
Seahorse 1 (Male 17)								
15 mins before feed (am)	180	720			#11		(AL)(AS)(HP)	
15 mins after feed (am)	540	360			#28		(AL)(AS)(HP)	
15 mins before feed (lunch)	467	433			#19		(HP)	
15 mins after feed (lunch)	574	326		Snapped Snout #8	#22		(HP)	
15 mins before feed (pm)	636	264			#13		(HP)	
15 mins after feed (pm)	900				#13		(AL)(AS)	
Seahorse 2 (Female)								
15 mins before feed (am)	900				#13		(AL)(AS)	
15 mins after feed (am)	900				#18		(AL)(AS)	
15 mins before feed (lunch)	900				#16		(AL)	
15 mins after feed (lunch)	900		#1		#21		(AL)	
15 mins before feed (pm)	900				#9		(HP)(AS)	
15 mins after feed (pm)	900				#1		(AL)(AS)	
Seahorse 3 (Male 13)								
15 mins before feed (am)	675	225			#27		(HP)	
15 mins after feed (am)	616	284			#21		(AL)(AS)(HP)	
15 mins before feed (lunch)	900				#18		(HP)	
15 mins after feed (lunch)	900			Snapped Snout #8	#20		(HP)	
15 mins before feed (pm)	900				#12		(HP)(AS)	
15 mins after feed (pm)	900				#7		(AL)(AS)	
Tuesday 23rd July 2013								
Seahorse 1 (Male 17)								
15 mins before feed (am)	335	565			#19		(AL)(AS)(HP)	
15 mins after feed (am)	270	630	#1		#19		(AL)(AS)(HP)	
15 mins before feed (lunch)	471	429			#12		(AL)(AS)(HP)	
15 mins after feed (lunch)	664	236		Wrestled #2 Snapped Snout #6	#21		(AL)(AS)	
15 mins before feed (pm)	900				#18		(AL)(AS)	Inflated Pouch #1
15 mins after feed (pm)	900						(AL)(AS)	Inflated Pouch #1
Seahorse 2 (Female)								
15 mins before feed (am)	900				#13		(AL)(AS)	
15 mins after feed (am)	900				#18		(AL)(AS)	
15 mins before feed (lunch)	900				#12		(AL)(AS)	
15 mins after feed (lunch)	900				#9		(AL)(AS)	
15 mins before feed (pm)	900				#6		(AL)(AS)	
15 mins after feed (pm)	900						(AL)(AS)	
Seahorse 3 (Male 13)								
15 mins before feed (am)	500	400			#24		(AL)(AS)HP)	
15 mins after feed (am)	510	390			#22		(HP)(IP)	
15 mins before feed (lunch)	675	225			#17		(AL)(AS)	
15 mins after feed (lunch)	867	33		Wrestled #2 Body Barge #1	#17		(AL)(AS)	
15 mins before feed (pm)	900			Snapped Snout #4	#9		(AL)(AS)	
15 mins after feed (pm)	900				#8		(HP)	
Wed 24th July 2013								
Seahorse 1 (Male 17)								